CHARACTERIZATION OF BACTERIAL COMMUNITIES AND POTENTIAL PATHOGENS IN FIELD-APPLIED BIOSOLIDS IN NORTHWEST OHIO

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ABSTRACT

The application of biosolids (sludge resulting from waste water treatment plants [WWTP] processing) to agricultural fields is a common practice in Ohio. Biosolids contain a vast variety of potential human pathogens, which may affect the community surrounding the fields. However, the microbial composition of biosolids might be variable depending on treatment regimes adopted in WWTPs, and is largely uncharacterized. The purpose of this study was to characterize the composition of bacterial communities and putative pathogens in biosolids generated from several WWTPs. Samples were analyzed for heterotrophic bacteria using R2A media, total coliforms and *Escherichia coli using* Rapid *E. coli* 2 media and *Staphylococcus spp.* using Baird Parker media. The results show that the number of bacteria and putative pathogens are significantly dependent on type of digestion treatment. Specifically heterotrophic bacteria (190,000 CFUs g-1 vs. 1,796,667 CFUs g-1), fecal coliforms (234,000 vs. 0 CFUs g-1), *E. coli* (3,334 vs. 0 CFUs g-1), and *Staphylococcus spp.* (68,000 vs. 3,166,667 CFUs g-1) fluctuated significantly (t-test) when comparing biosolids from two different class B WWTP. Most WWTPs inspected showed a decrease in fecal coliforms and *E. coli* numbers, while *Staphylococcus spp.* numbers tended to increase. To conclude, the treatments adopted in class B biosolid WWTP might impact gram negative bacterial pathogens (*E. coli*) but not gram positive bacteria (*Staphylococcus spp.*), because they are more resistant. However, not all *Staphylococcus spp.* are pathogenic, which warrants further investigation into the specific pathogenic *Staphylococcus spp.* (*S. aureus*) using DNA fingerprinting techniques such as denaturant gradient gel electrophoresis.

BACKGROUND

Biosolids are defined by the Environmental Protection Agency as nutrient-rich organic materials resulting from the treatment of domestic sewage in a treatment facility. Following treatment, these residuals can be recycled and applied as soil conditioners to improve the physical and chemical conditions of the soil, thereby maintaining productive soils and stimulated plant growth (1).

Under EPA standards, biosolids must meet requirements for minimizing or eliminating pathogens and reducing pathogen vector attraction. For pathogens, these requirements can be met by reducing the pathogens in biosolids to below detectable levels or to levels that are reduced but still detectable and are coupled with certain restrictions (2). Despite these measures, of current concern is the impact of biosolids application on public health, water quality, and the impact of biosolids application of the indigenous soil community.

A few of the human pathogens associated with biosolids are *Escherichia coli, Salmonella sp., Shigella sp., Campylobacter jujuni* and *Staphylococcus sp.* (2). Humans are potentially exposed to these pathogens through direct contact with biosolids or indirect contact by consumption of contained foods. Unfortunately, an insufficient amount of research has been performed on biosolids designated for land-application to characterize the potential harm of biosolids.

OBJECTIVES

- Characterize the changes in the activity and structure of agricultural soil bacteria as a result of biosolids application.
- Determine the fate of selected pathogens in biosolids following application to agricultural soils.
- Determine if a history of biosolids application impacts the response of the soil community to subsequent biosolids applications.

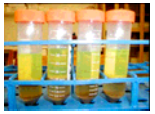
METHODS

- Soil collected in microcosms from Bowling Green Municipal Fields (with history and no history of application) were spiked with biosolids from the Oregon WWTP. The microcosms were stored in an incubator at 25°C and monitored weekly for ten weeks as follows:
- Plate count analyses were performed to enumerate the total heterotrophic bacteria total coliforms, *E. coli*, and staphylococci.
- The fluorescein diacetate (FDA) hydrolysis assay was used to assay overall microbial metabolic activity. Upon hydrolysis due to enzymatic activity, colorless FDA breaks down into fluorescein, resulting in green coloration. A spectrophotometer was used to measure the intensity of the coloration and thus the relative microbial activity in the treated soils.
- Community level physiological profiling (CLPP) was used to estimate metabolic diversity in the treated soils. Diluted soils were inoculated into microplates that contained several single carbon sources in addition to a tetrazolium dye. The utilization of any carbon source by the community results in the reduction of the dye and purple color formation that can be quantified and monitored over time (3).
- Community structure was assessed through DGGE analysis of PCR amplified rDNA fragments. This analysis provided information on the structure of the microbial communities in the biosolids, soils, and biosolids-amended soils.

Microbial Activity

Fluorescein Diacetate Hydrolysis Assay

Microbial activity results in a quantifiable amount of fluorsecein (fluorescent yellow-green) resulting from the hydrolytic cleavage of FDA (colorless).



Example of a FDA reaction. The intensity of the green color indicates the metabolic usage of Fluorescein by the community.

CLPP

Tests the ability of microorganisms to metabolize 31 carbon substrates preformed in triplicate. Plates were incubated at 25°C for 72 h and then scanned with a spectrophotometer to determine carbon use patterns.

Model 680 Bio-Rad Microplate Reader used to scan CLPP microplates.

Bacteria Enumeration

Dilution plating

Rapid *E. coli* II media was used for the detection/counts of total coliforms (most will appear blue) and *E. coli* (most will appear purple). Plates were incubated at 35°C for 24 h.

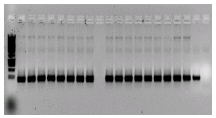
R2A agar was used for the detection/counts of heterotrophic bacteria. Plates were incubated at 25°C for 72 h.

Baird Parker media w/ egg yolk-tellurite enrichment was used for the detection/counts of *Staphylococcus spp.* Plates were incubated at 35°C for 48h. (*S. aureus* will grow black, accompanied by a halo)

Microbial Community Structure

Polymerase Chain Reaction (PCR)

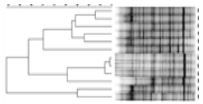
16S rDNA PCR was used to generate *Bacteria* domain DNA fragments to be separated by DGGE.



PCR product on a 1% agarose gel.

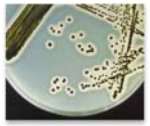
Denaturing Gradient Gel Electrophoresis (DGGE)

A molecular fingerprinting method that separates (PCR)-generated DNA Products.



Results from DGGE after computerized dendrogram analysis.

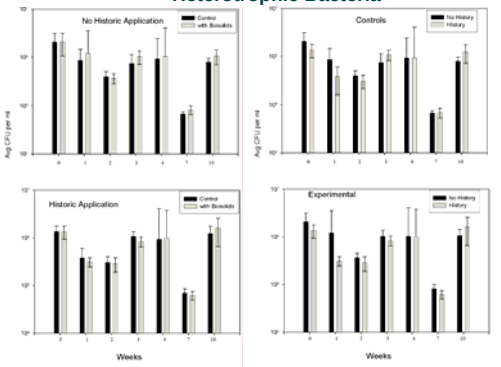
Inner chamber that contains the DGGE gel.



S. aureus on Baird Parker media w/ tellurite enrichment.

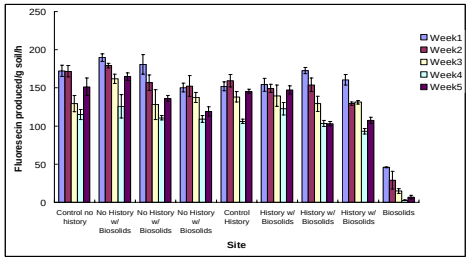
RESULTS

Enumeration of Soil Samples from Bowling Green Municipal Fields for Total Heterotrophic Bacteria



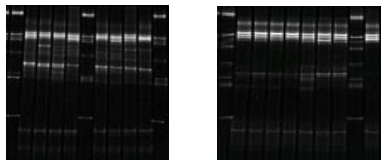
CFU's for both control and experimental microcosms showed correlating increases and decreases over the duration of the experiment, but returned to the initial level by the tenth week. The biosolids treatment resulted in no significant differences.

FDA Analysis



FDA analysis showed both increases and decreases in metabolic activity of the bacterial community throughout the duration of the experiment. Fluorescein production correlated with the numbers of total heterotrophic bacteria in the soils. In the fifth week signs of re-growth were observed in the biosolids.

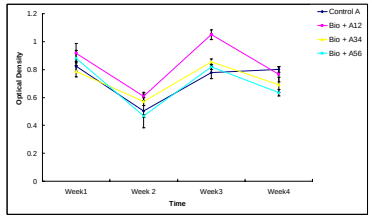
DGGE Analysis



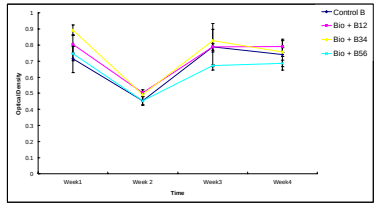
No major structural differences in community structure were detected following DGGE of DNA isolated from soils collected during Week 1(left) and Week 7(right).

CLPP Analysis

Average Metabolic Response (no History of biosolids application)



Average Metabolic Response (history of biosolids application)



CLPP analysis showed a decrease then an increase in microbial metabolic activity.

CONCLUSIONS

- The impact of biosolids application on soil microbial communities appears to be nonsignificant.
- Enumeration analysis showed a fluctuation in the soil's total heterotrophic bacteria, but returned to almost normal levels by the tenth week.
- Although metabolic activity followed a similar pattern of fluctuation, the levels of fluorescein production decreased throughout the duration of the project.
- DGGE analyses are still being performed. However, our results thus far indicate that biosolids treatment has little impact on the soil microbial community.

References

(1) Hickman, John S. and Whitney, David A. Soil Conditioners. (North Central Regional Extension Publication 295. Department of Agronomy, Kansas State University.

(2) United State Environmental Protection Agency. Environmental Regulations and Technology. (Office of research and Development Washington, DC 20460. EPA/625?R-92/013 Revised October 1999 http://www.epa.gov/ORD/NRMRL

(3) Laboratory for Microbial Ecology. Department of Earth, Ecological and Environmental Sciences, University of Toledo (http://www.eeescience.utoledo.edu/Faculty/Sigler/RESEARCH/Protocols/CLPP/CLPP.pdf)